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1 **Biocide resistance and transmission of *Clostridium difficile* spores spiked onto clinical**
2 **surfaces from an American healthcare facility**

3

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10

11 Running Head: Transmission and resistance of *C. difficile* spores

12 **Abstract**

13 *Clostridium difficile* is the primary cause of antibiotic-associated diarrhea globally. In
14 unfavourable environments the organism produces highly resistant spores which can survive
15 microbicidal insult. Our previous research determined the ability of *C. difficile* spores to adhere
16 to clinical surfaces; finding that spores had marked different hydrophobic properties and
17 adherence ability. Investigation into the effect of the microbicide sodium dichloroisocyanurate
18 on *C. difficile* spore transmission revealed that sub-lethal concentrations increased spore
19 adherence without reducing viability. The present study examined the ability of spores to
20 transmit across clinical surfaces and their response to an in-use disinfection concentration of
21 1,000-ppm of chlorine-releasing agent sodium dichloroisocyanurate. In an effort to understand
22 if these surfaces contribute to nosocomial spore transmission, surgical isolation gowns,
23 hospital-grade stainless steel and floor vinyl were spiked with 1×10^6 spores/ml of two types
24 of *C. difficile* spore preparations: crude spores and purified spores. The hydrophobicity of each
25 spore type versus clinical surface was examined via plate transfer assay and scanning electron
26 microscopy. The experiment was repeated and spiked clinical surfaces were exposed to 1,000-
27 ppm sodium dichloroisocyanurate at the recommended 10-min contact time. Results revealed
28 that the hydrophobicity and structure of clinical surfaces can influence spore transmission and
29 that outer spore surface structures may play a part in spore adhesion. Spores remained viable
30 on clinical surfaces after microbicide exposure at the recommended disinfection concentration
31 demonstrating ineffectual sporicidal action. This study showed that *C. difficile* spores can
32 transmit and survive between varying clinical surfaces despite appropriate use of microbicides.

33 **IMPORTANCE**

34 *Clostridium difficile* is a healthcare-acquired organism and the causative agent of antibiotic-
35 associated diarrhoea. Its spores are implicated in faecal to oral transmission from contaminated

36 surfaces in the healthcare environment due to their adherent nature. Contaminated surfaces are
37 cleaned using high-strength chemicals to remove and kill the spores; however, despite
38 appropriate infection control measures, there is still high incidence of *C. difficile* infection in
39 patients in the US. Our research examined the effect of a high-strength biocide on spores of *C.*
40 *difficile* which had been spiked onto a range of clinically relevant surfaces including isolation
41 gowns, stainless steel and floor vinyl. This study found that *C. difficile* spores were able to
42 survive exposure to appropriate concentrations of biocide; highlighting the need to examine
43 the effectiveness of infection control measures to prevent spore transmission, and consideration
44 of the prevalence of biocide resistance when decontaminating healthcare surfaces.

45 **Introduction**

46 The anaerobic spore-forming Gram-positive bacterium *Clostridium difficile* is the primary
47 cause of antibiotic-associated diarrhea globally (1). *C. difficile* asymptotically forms part of
48 the microbiota of 1-3% healthy adults (2, 3); however, if the microbiota of the intestine is
49 disrupted, for example as a result of broad- spectrum antibiotic treatment, colonisation of the
50 colon by vegetative cells of *C. difficile* can proceed and escalate into the onset of *C. difficile*
51 infection (CDI) (4). When fulminant infection ensues the patient will suffer from inflammation
52 and diarrhoea. Further complications of CDI include pseudomembranous colitis, sepsis and the
53 fatal toxic megacolon (5).

54 Hypervirulent PCR ribotypes such as BI/027/NAP1 have spread intercontinentally and caused
55 epidemics in Western countries further adding to CDI incidence (6, 7). Many reports highlight
56 the increasing impact of CDI to public health and the associated economic burden. For
57 example, mortality rates in the United States increased from 25 to 57 per million people for the
58 periods 1999-2000 and 2006-2007, respectively (8). In total approximately 14,000 deaths
59 occurred in 2007 and this statistic increased still further with an estimated 29,300 deaths in

60 2011 (9). In 2008 alone the estimated cost related to CDI within the United States to health-
61 care facilities was \$4.8 billion, ignoring the additional cost to other facilities such as care homes
62 (10). A similar pattern of statistics can be seen in England, with an increase from 1,149 *C.*
63 *difficile*-related deaths in 2001 rising to 7,916 in 2007 (11).

64 In response to increasing CDI infection rates, stringent infection control procedures were
65 implemented within hospital environments in England which resulted in a decline in mortality
66 to 1,487 in 2012. This figure surpasses that of MRSA and non-specified *Staphylococcus aureus*
67 infection mortality (262 in 2012) (12) and thus is still a major source of concern globally.
68 Despite implementation of appropriate surveillance and infection control procedures the
69 organism still causing significant levels of morbidity and mortality across nosocomial
70 environments (13).

71 Incidence of CDI is directly affected by the ability of *C. difficile* to produce resistant spores
72 which can survive on organic and inorganic surfaces for months and remain viable (6). A major
73 source of CDI and transmission in the healthcare environment is through the faecal to oral
74 route; often via the contamination of surfaces. As many as 1×10^7 spores per gram faeces are
75 released into the environment by infected patients through airborne dispersal and soiling further
76 adding to the bioburden (14). Possible causes of transmission include inappropriate biocide
77 use, lack of adherence to infection control guidelines and varying standards of practice across
78 healthcare facilities globally (15, 16, 17).

79 Chlorine-releasing agents (CRAs) are the predominant form of biocide used in healthcare
80 facilities to disinfect surfaces; namely sodium hypochlorite (NaOCl) and sodium
81 dichloroisocyanurate (NaDCC) (18). These microbicides are fast-acting in aqueous solutions
82 and are relatively inexpensive (19). Low concentrations of 50-ppm available chlorine have
83 shown to kill >99% of vegetative bacterial cells *in vitro*. In addition, when 275-ppm chlorine

84 was applied to a clinical environment there was a significant reduction in hospital-acquired
85 infections from non-spore forming bacteria (20, 21). However, the inactivation of spores
86 requires much higher concentrations with the current recommendation for application of
87 NaDCC in hospitals in England being 1,000-ppm available chlorine for 10 minutes to
88 deactivate spores of *C. difficile* and *Bacillus* species (22, 23). Although the working
89 concentration of NaDCC has shown to be effective in liquid culture (24), its application to
90 working surfaces is less efficient for inactivation of spores (25) and this reduced activity is
91 exacerbated by the presence of organic substances, such as bodily fluids and faeces, which
92 have a neutralising effect on the biocide (26). The mechanism of action of chlorine-releasing
93 biocides is poorly understood; however, it has been suggested that their action may be due to
94 strong oxidative ability, their effect on cell membranes and inhibition of enzymatic reactions
95 (27).

96 Our previous study showed that adherence of *C. difficile* spores to inorganic surfaces increased
97 when spores were exposed to sub-lethal concentrations (500-ppm available chlorine) of sodium
98 dichloroisocyanurate (27). This increase was more pronounced for strain DS1748 (002
99 ribotype) which is not known to produce an exosporium outer layer (28) and suggests that when
100 spores are exposed to sub-lethal levels of biocide they may inadvertently become more
101 adherent to inorganic surfaces. The purpose of the present study was to assess the transfer
102 ability of *C. difficile* spores from clinical surfaces pre- and post-biocide exposure. Surfaces
103 tested include hospital isolation gowns, hospital grade stainless steel and vinyl flooring
104 routinely used within the United States. Spore recovery from spiked clinical surfaces was
105 investigated using a plate transfer assay. Clinical surfaces spiked with spores were exposed to
106 NaDCC to determine sporicidal efficacy and the presence of spores on each clinical surface
107 pre and post NaDCC treatment was examined using scanning electron microscopy.

108 **Results**

109 **Transfer of *C. difficile* spores from liquid form to hospital surgical gowns**

110 To examine the ability of *C. difficile* spores (U and P derived from strains DS1748, R20291
111 and DS1813) to adhere to, and subsequently transfer from hospital surgical gowns, spores were
112 applied directly to the surgical gowns in liquid for 10 s, 30 s, 1 min, 5 min and 10 min before
113 being removed and discarded (Figure 1, Figure 4A and C). This experiment was designed to
114 mimic transfer of infectious bodily fluids in the clinical setting and assess the potential for
115 onward transmission to patients. There was no significant difference between the amount of
116 spores (U and P) recovered from the gowns and the contact time of the spores to the gowns;
117 suggesting that the process of spore transfer between surfaces occurred within the first 10
118 seconds of contact with the gown (two-way ANOVA; $p = 0.696$). From Figure 1 it appears as
119 though the recovery of DS1748 P Spores increased with contact time; however, this was not
120 statistically significant (one-way ANOVA; $p = 0.144$). Generally, U spore recovery was
121 significantly higher than that of P spores (two-way ANOVA; $p < 0.001$); however, the
122 exception to this trend was the increased recovery of DS1813 P spores when compared to U
123 spores of the same strain (one-way ANOVA; $p < 0.001$). There were no significant differences
124 in spore recovery between DS1748 and R20291 for either U spores or P spores.

125

126 **Spore recovery from spiked clinical surfaces after direct contact with hospital gowns**

127 To establish whether hospital-grade stainless steel surfaces and vinyl flooring surfaces act as
128 fomites for *C. difficile* spore transmission in the clinical setting, sterile sections of hospital
129 surgical gowns were placed in direct contact with hospital-grade stainless steel and vinyl
130 flooring spiked with 1×10^5 spores and spore recovery from the surgical hospital gowns
131 assessed. The contact times were reduced to 10 s, 30 s and 1 min due to results presented in
132 Figure 1 which confirm that the length of contact time had no significant effect on spore
133 recovery. Similarly, there remained no significant difference in spore recovery from steel and

134 vinyl between the contact times used and the amount of spores recovered from the strains
135 examined (Figure 2) (two-way ANOVA; $p = 0.892$ and $p = 0.904$ for steel and vinyl,
136 respectively). Spore recovery of U DS1748 was significantly higher from both stainless steel
137 surfaces (one-way ANOVA; $p = 0.034$) and vinyl flooring (one-way ANOVA; $p < 0.001$) when
138 compared to the other strains. DS1748 P spore recovery was higher on stainless steel (one-way
139 ANOVA; $p < 0.001$) and vinyl flooring (one-way ANOVA; $p < 0.001$) than of R20291 and
140 DS1813. DS1748 P spore recovery was approximately 10-fold higher than that of the U Spore
141 equivalent (two-way ANOVA; $p < 0.001$).

142

143 **Sporicidal efficiency of sodium dichloroisocyanurate (NaDCC)**

144 Two types of spore suspension from three *C. difficile* strains (DS1748, R20291 and DS1813)
145 were exposed to the recommended in-use concentration of NaDCC in solution (1,000-ppm)
146 and spore viability was determined. From Figure 3 it can be seen that there was no recovery of
147 spores which had been treated in liquid form and then spiked onto gowns. Moreover, recovery
148 of NaDCC-treated U spores from the spiked and directly-treated hospital surgical gowns were
149 lower across the three strains tested when compared to non-treated spores, with the lowest
150 relative recovery from strain R20291 (Student t-test; $p < 0.005$). Scanning Electron Microscopy
151 (SEM) images in Figure 4A and 4C support this by showing adhered spores on the fibres of
152 the gowns from strain R20291 before and after treatment with the recommended concentration
153 of NaDCC. Interestingly, Figure 4B shows a single P spore of R20291 after NaDCC treatment
154 with a visible exosporial layer, while Figure 4D shows a U spore of R20291 after treatment
155 that has no visible evidence of an exosporial layer. These differences in spore exosporium show
156 distinct morphological variations within the R20291 strain; but may not necessarily be as a
157 result of NaDCC exposure. It is possible that any damage to the exosporium after NaDCC
158 exposure is not visible via SEM (Figures 4B); thus there is a possibility that NaDCC may have

159 chemically altered the exosporium structure without changing the spore's overall three-
160 dimensional appearance (28).

161 Decreased sporicidal activity was observed for strains tested with NaDCC on the varying
162 clinical surfaces (Figure 3). Similar results were observed with DS1813 P spores, but not for P
163 spores of DS1748 and R20291. There was detectable recovery of R20291 U Spores (~73 to
164 ~23 SFU) after NaDCC treatment on stainless steel; although this was not significantly
165 different when compared to the lack of recovery of the other U strains tested (Mann-Whitney
166 Test; $p = 0.40$). Despite the lack of DS1813 spore recovery from stainless steel surfaces after
167 NaDCC exposure (Figure 3C), spores were still present on the steel surfaces indicating lack of
168 viability (Figure 5A).

169 After NaDCC exposure no DS1748 or R20291 P spores were recovered from the vinyl flooring,
170 whereas U spores from these strains were recovered (Figure 3A and B). SEM results revealed
171 the presence of spores of both types on the vinyl (Figure 5B and D). The recovery of R20291
172 U spores significantly decreased (Student's t-test; $p = 0.001$) but not for DS1748 (Figure 3A
173 and B). In contrast, the recovery of both U and P spores of DS1813 did not change significantly
174 after NaDCC treatment (Student's t-test; $p > 0.05$ for both U spores and P spores; Figure 3C).

175 **Discussion**

176 Gowns have been used by healthcare professionals to mitigate the risk of transmitting
177 infectious materials between patients, hospital visitors and other healthcare workers (31). Many
178 gowns have shown differences in barrier and textile performance and it is these variations that
179 play a role in the dissemination of microorganisms across healthcare facilities (32). With the
180 advent of modern technology single-use isolation gowns made from fluid-resistant materials,
181 such as polypropylene, are now widely used as a form of barrier protection; however, there is
182 some debate as to their efficiency (31, 33, 34). Our results demonstrated that *C. difficile* spores

183 were able to transfer and adhere to fibres of the polypropylene spun gowns when spiked in a
184 liquid medium. As there was no significant difference between the contact time of the spores
185 and the recovery of spores from the gown, it appears as though the process of spore transfer
186 occurred rapidly within the first 10 seconds of contact when examining spore recovery from
187 spiked liquid, hospital grade stainless steel and vinyl flooring, respectively. This suggests a
188 clear need to ensure appropriate decontamination of surfaces that a contaminated gown may
189 come into direct contact with in a clinical setting.

190 The ability of microorganisms to travel through fabrics is related to the physico-chemical
191 properties of the gowns and the characteristics of the microorganism (32). Another interesting
192 observation from this study is the rapid ability of the spores to move from one hydrophobic
193 surface to another hydrophobic surface i.e. fluid-resistant gowns and stainless steel which
194 suggests that the more hydrophobic spores interacted better with the stainless steel surfaces
195 than the gowns (Table 1, Figure 1). Whether this is related to steel surface structure as opposed
196 to gown structure, or the hydrophobic interactions between (i) the individual strains (which
197 possess varying relative hydrophobicity; Table 1) (ii) the liquid and (iii) each test surface
198 warrants further investigation at the molecular level. It is also clear that the single-use gowns
199 act as fomites for *C. difficile* spore transmission. Not only do spores of all strains rapidly attach
200 to the gown fibres from liquid and dry clinical surfaces but the single-use gowns are then
201 ineffective at trapping spores within their fibres and preventing the onward transmission of
202 spores as demonstrated by spore recovery from the gowns (Figures 1& 2). While this ability
203 differs between strains, it does suggest that the adherence ability of the spore to individual
204 gown fibres may be affected by spore hydrophobic properties and exosporium layer which is
205 known to aid spore adherence on hospital surfaces (Table 1; 28). Results also suggest that *C.*
206 *difficile* spores, after microbicidal exposure to NaDCC at the recommended contact time and
207 concentration, can continue to remain viable, adhere and transmit via hospital gowns (Figure

208 4A & 4C; 1, 28, 35). This highlights the importance of ensuring that single-use surgical
209 isolation gowns are used appropriately in infection prevention and control; i.e. that gowns are
210 adorned upon entering and disposed of when exiting a single room to prevent onward spore
211 transmission and incidence of CDI (36).

212 Despite using recommended concentrations of NaDCC to decontaminate gowns, stainless steel
213 surfaces and floor vinyl after spore exposure, spores were still visibly attached to each surface
214 and were viable upon culture (Figures 4 & 5). Decontamination and appropriate cleaning of
215 surfaces is critical in managing the spread of CDI to patients from spores (37). It can be
216 speculated that the hydrophobic properties and weave of the gown fabric may have prevented
217 exposure of spores to NaDCC which explains the increased spore recovery; however this would
218 need to be examined further by exploring the use of fluorescence-based spore viability tests
219 (38). The smooth surfaces of steel and vinyl would theoretically make NaDCC treatment more
220 effective by increasing the test surface area; however, the occurrence of viable spores on both
221 treated steel and vinyl surfaces conflicts with this hypothesis and clearly evidences spore
222 resistance to NaDCC. This resistance was found for all three strains tested and was not limited
223 to hypervirulent R20291 027 PCR ribotype strains (7) (Table 1). Our results confirm that
224 working concentrations of sporicides (with active concentrations of chlorine) applied at the
225 appropriate contact times may not kill *C. difficile* spores. The ability of microbicides, such as
226 CRA's, to kill *C. difficile* spores has been examined previously with similar results (7, 25, 26,
227 38).

228 Spores which possess an exosporium-like structure have been demonstrated to have increased
229 adhesion to surfaces *in vivo* and *in vitro*; associated with increased hydrophobicity of the spore
230 (28, 35, 39). The exact function of the exosporium-like structures on certain strains of *C.*
231 *difficile* spores has yet to be fully elucidated; however, its role in adhesion to intestinal mucosal
232 cells and in *Bacillus* spore adhesion has been more clearly defined (35, 39, 40). Our previous

233 study established that exosporium-positive spores (DS1813) were more resistant to NaDCC at
234 sub-lethal concentrations than exosporium-negative spores (DS1748) (1, 28), which appears to
235 correlate with the theory that the exosporium layer confers a protective barrier to the spore,
236 preventing it from being damaged (41). It has also been hypothesised that exposure of spores
237 to NaDCC at inappropriate concentrations and contact times can alter and increase spore
238 adhesion ability (1). In the present study, while we observed a lack of exosporium-negative
239 DS1748 and exosporium-positive DS1813 spore recovery from hospital stainless steel, SEM
240 image (Figure 5A) revealed the presence of DS1813 spores adhered onto the stainless steel
241 surface, and the presence of possible damaged spores of DS1748 (Figure 5C). Indeed, the
242 presence of a small number of spores following NaDCC treatment could still produce recovery
243 of zero viable spores. Moreover, the viability of spores from all strains tested was also observed
244 after NaDCC treatment of vinyl flooring (Figure 3). This strongly indicates that recovery of
245 spores from stainless steel and vinyl, two very different materials, has been affected by biocide
246 exposure, either due to biocidal killing or reduced spore adherence; however, the exact
247 mechanism of spore adherence and biocidal activity of NaDCC upon the exosporium layer and
248 spore ultrastructure has yet to be determined.

249 As seen in Figures 4 and 5, there are exosporium-like projections present on R20291 spores
250 that increase the material surface-spore contact area which correlates to data from other studies
251 (41). It is possible that these projections may increase spore adherence and perhaps biocide
252 resistance by protecting the core from chemical effects. Moreover, as NaDCC was completely
253 effective when spores were exposed in liquid form (Figure 3) when compared to the spore
254 recovery post exposure from spiked surfaces, attests to the potential the exosporium may have
255 for protection of the spore from biocide exposure. Interestingly, hypervirulent DS1813 and
256 R20291 strains have shown an increased adherence ability throughout this study comparative
257 to DS1748; suggesting exosporium- positive spores adhere better and more rapidly with first-

258 contact to the test surface (Table 1). Additionally, unpurified R20291 spores were recovered
259 from all surfaces tested post-NaDCC exposure which demonstrates the spore's ability to remain
260 viable after biocide exposure (Fig 2 & 3). This concurs with previous studies that have
261 demonstrated CRA resistance in PCR Ribotypes 017, 012 and 027 (R20291) (7). Mechanical
262 removal to remove spores from clinical surfaces has been shown to be effective in studies,
263 however, this may not be appropriate with gowns as they are designed for single-use; therefore
264 effective and immediate disposal of surgical gowns after use needs to be considered when
265 preventing transmission of CDI (6, 25). The impact of the microbicide upon spore structure
266 and resistance warrants further research to fully understand the mechanisms of resistance and
267 to establish up-to-date and effective decontamination protocols. Moreover, our research
268 suggests that the *C. difficile* exosporium may play a key role in biocide resistance of spores and
269 thus could be a potential target for development of novel sporicidal disinfectants.

270 **Materials and Methods**

271 **Growth conditions, *Clostridium difficile* strains and spore production**

272 *C. difficile* cultures were incubated anaerobically at 37 °C for 48 hours in a BugBox Plus
273 anaerobic workstation (Ruskinn Technology Ltd. United Kingdom) using an 85% nitrogen,
274 10% carbon dioxide and 5% hydrogen gas mix. Clinical isolates of *C. difficile* (PCR Ribotypes
275 027 and 002) used in this study are described in Table 1 and were obtained from the Anaerobic
276 Reference Unit, University Hospital Wales, Cardiff, UK. Unless otherwise stated, all
277 organisms were stored as spores at 4 °C. All experiments described were conducted in
278 triplicate. *C. difficile* spores were produced according to two methods to generate
279 unpurified/crude and purified spore preparations; spores produced *via* Perez et al 2005 (42)
280 methodology were designated as unpurified (U) spores due to being harvested via water-
281 washing and containing vegetative and spore forms of the organism. These were deemed

282 representative of *C. difficile* commonly encountered within clinical environments. Spores were
283 produced on reduced brain heart infusion (BHI) agar and BHI broth (Oxoid Ltd, Basingstoke,
284 United Kingdom) each supplemented with the germinant 0.1% (w/v) sodium taurocholate (28).
285 Purified spores (P spores) were produced as described by Sorg and Sonenshein (2010) (43).
286 Briefly, *C. difficile* strains were cultured on reduced BHI agar with 5 g/L yeast extract and
287 0.1% L-cysteine and were examined after four days anaerobic incubation for characteristic
288 colonies. Spores were harvested using sucrose density-washing. Spore purity was confirmed
289 via phase contrast microscopy. Spore concentration was determined via drop count as described
290 by Miles *et al.* (44) and mean spore-forming units (SFU) per ml calculated (28).

291 **Preparation of clinical surfaces**

292 Single-use hospital surgical gowns were produced by MediChoice, order no. 77752XL (45),
293 made from fluid-resistant spunbond-meltdown-spunbond (SMS) polypropylene laminate at
294 AAMI PB70:2012 (46) standard at level 2. To test the transfer of spores to and from the gowns,
295 gowns were aseptically cut into 7×7 cm sections and testing performed within a drawn circle
296 of 2 cm diameter to confer with the surface area of the hospital grade 2B stainless steel discs
297 and vinyl flooring used in this study.

298 **Spore Transfer to Hospital Surgical Gowns**

299 To test the number of spores transferred to the hospital surgical gown after direct contact, U
300 Spores and P Spores from strains DS1748, DS1813 and R20291 (Table 1) were produced at 1
301 $\times 10^4$ spores/ml. From these, 100 μ l were spiked onto the gown surface in triplicate experiments
302 and allowed to remain in static contact for 10 s, 30 s, 1 min, 5 min and 10 min before being
303 removed and discarded. After contact with spores, each section of gown was aseptically
304 mounted onto a plunger pre-affixed with a steel disc so that the disc was aligned with the test

305 area. A plate transfer test was then performed as described in Joshi *et al.*, (28). A force of 100g
306 was used as a simulated “touch” pressure.

307 **Spore Transfer from spiked “high-touch” surfaces to Hospital Surgical gowns**

308 To test the number of spores transferred to the surgical hospital gown from dry “high-touch”
309 surfaces (hospital grade stainless steel and vinyl flooring), U and P spores were produced at
310 concentrations of 1×10^6 spores/ml. Sterilised hospital grade steel discs and vinyl flooring were
311 inoculated with 100 μ l of spores and allowed to dry completely for 120 min in a Category 2
312 Biosafety laminar flow cabinet. Sections of gown were then placed in contact with the steel
313 and vinyl under 100g pressure for 10 sec, 30 sec and 1 min and the gown was then pressed onto
314 the appropriate agar plate for 10 sec at 100 g pressure (28). All agar plates were then incubated
315 for 48 hrs at 37 °C under anaerobic conditions. Following incubation colonies were counted
316 and SFU per ml were calculated.

317 **Exposure of Spores to Sodium Dichloroisocyanurate disinfectant**

318 Spore suspensions (U and P) from strains DS1748, R20291 and DS1813 at a concentration of
319 1×10^6 spores per ml were exposed to 1000-ppm NaDCC for 10 minutes in liquid form
320 (recommended contact time), neutralised with sodium thiosulfate and deposited onto sterile
321 gowns. Spores were recovered as described previously (1, 22). Secondly, spores were also
322 spiked onto the gown surface, as described in the spore transfer section above, and spores were
323 spiked onto the surfaces of hospital stainless steel and hospital vinyl flooring, respectively, for
324 each biological repeat and allowed to dry for 120 min in a Category 2 Biosafety laminar flow
325 cabinet. The three spiked surfaces were then directly exposed to 100 μ l NaDCC at 1000-ppm
326 for 10 minutes and neutralised with 1% sodium thiosulphate before plate transfer experiments
327 were performed and spore recovery recorded. Three technical repeats of each experiment were

328 performed. Control experiments where spores were exposed to sodium thiosulfate, sterile
329 deionised water and NaDCC alone were also performed.

330 **Scanning electron microscopy**

331 Gowns, steel and vinyl were analysed using scanning electron microscopy for the presence of
332 characteristic spores before and after treatment with NaDCC. Spores which had not been
333 exposed to NaDCC were used as a comparative control. Test surfaces were sputter coated with
334 metal using a gold palladium sputtering target (60% Au and 40% Pd from Testbourne Ltd) and
335 argon as the sputtering gas. Images were taken on a scanning electron microscope (Zeiss Sigma
336 HD Field Emission Gun Analytical SEM) using an accelerating voltage of 5 kV. Over 100
337 individual spores were viewed per sample at magnifications of x 4, 890 and x 83,380.

338 **Statistical Analysis**

339 Data are expressed as means \pm SEM. Paired T-tests, One way ANOVA, 2-way ANOVA and
340 Mann-Whitney U tests were performed using Minitab 17.

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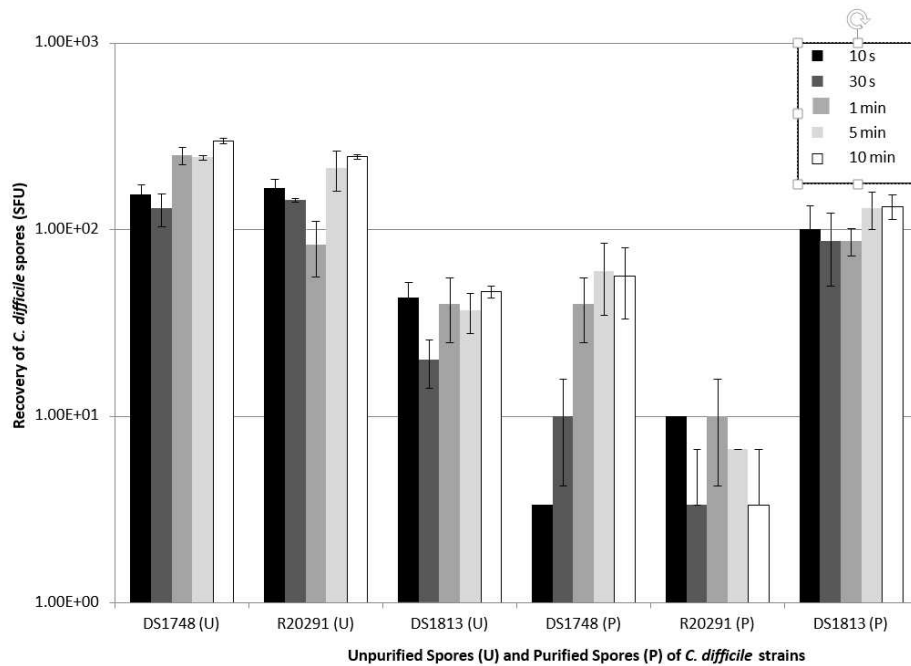
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498 **Tables: Table 1** *Clostridium difficile* strains used in the present study.

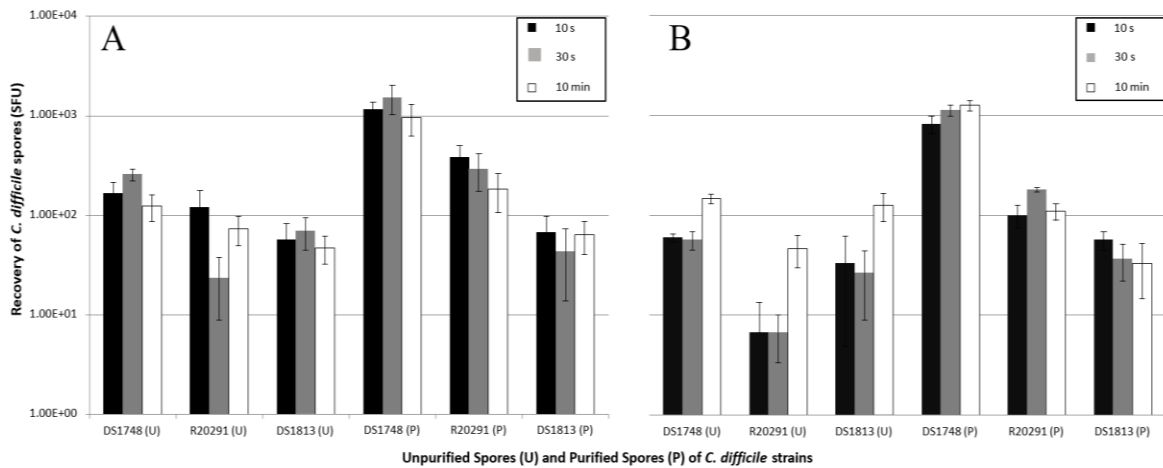
| <i>C. difficile</i> strain | PCR Ribotype | Source | Exosporium Presence | Relative Hydrophobicity |
|----------------------------|--------------|------------------|---------------------|-------------------------|
| DS1813 | 027 | Hinchingbrooke | Positive | 77% |
| R20291 | 027 | Stoke-Mandeville | Positive | 62% |
| DS1748 | 002 | Leeds | Negative | 14% |

499 **Figures & Legends**



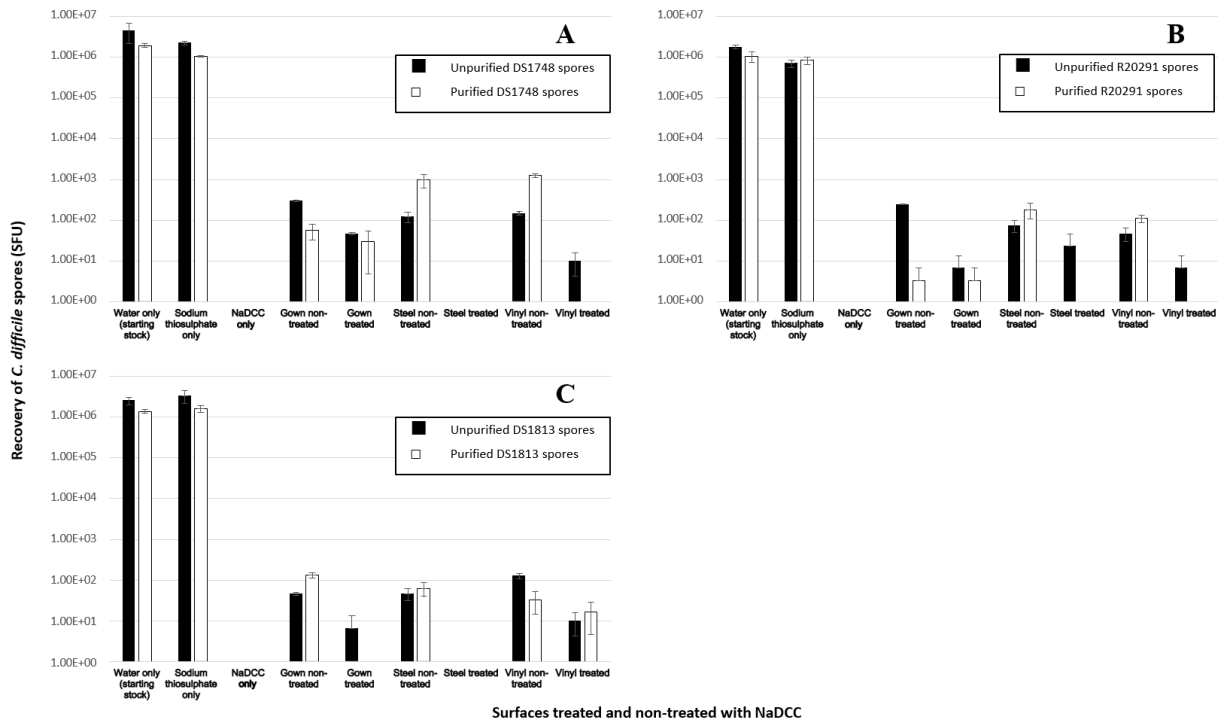
500
 501 Figure 1: Recovery of two different *C. difficile* spore types (Unpurified [U] and Purified [P])
 502 from spiked hospital surgical gowns. Spores were derived from strains DS1748, R20291 and

503 DS1813 and spores recovered after being exposed to the gowns at contact times ranging from
504 10 s to 10 min. Plots represent mean \pm SEM (n = 3).



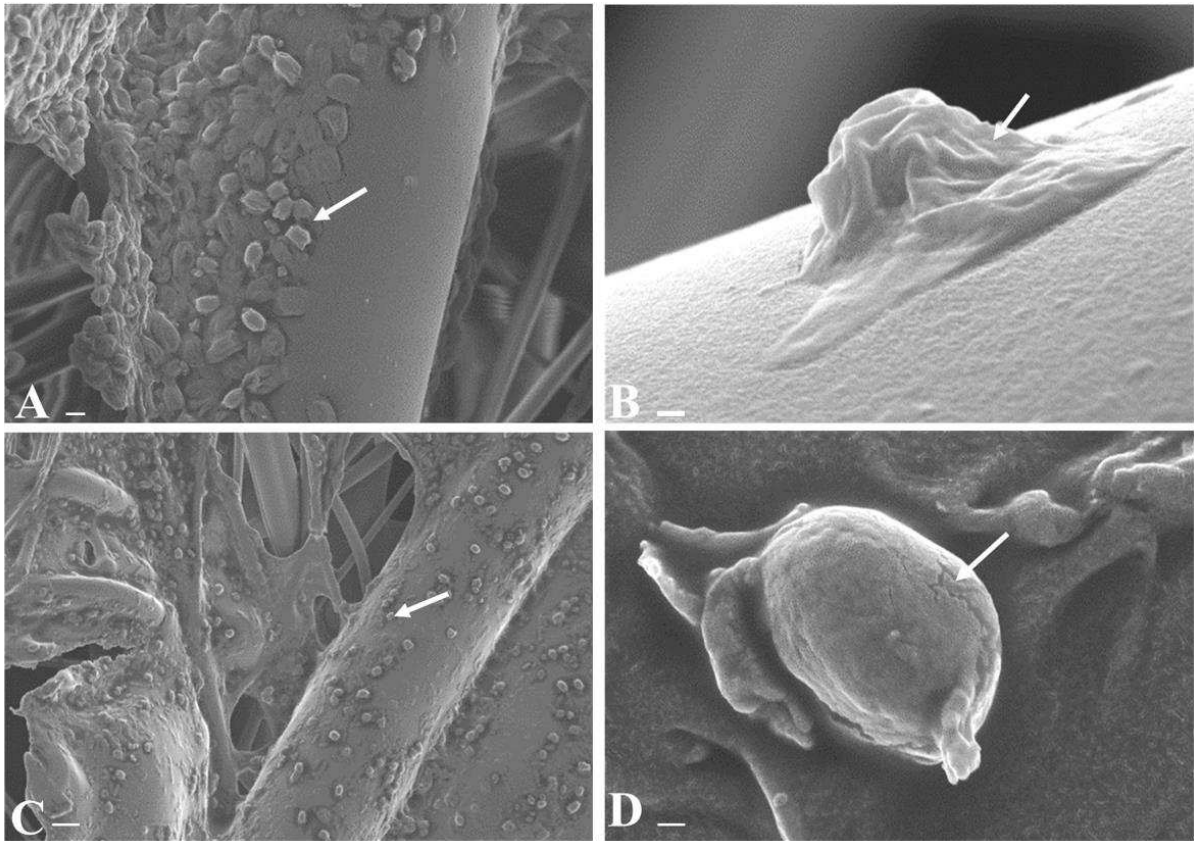
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506 Figure 2: Transmission ability of two different *C. difficile* spore types between clinical surfaces.
507 Spores derived from strains DS1748, R20291 and DS1813 were spiked onto hospital stainless
508 steel and vinyl surfaces and their ability to transfer to hospital surgical gowns was tested.
509 Unpurified (U) and purified (P) spores were recovered via transfer test from (A) hospital grade
510 stainless steel and (B) hospital vinyl flooring using hospital surgical gowns applied at a
511 pressure of 100g. Contact times ranged at 10s, 30 s and 1 min. Plots represent mean \pm SEM
512 (n = 3).



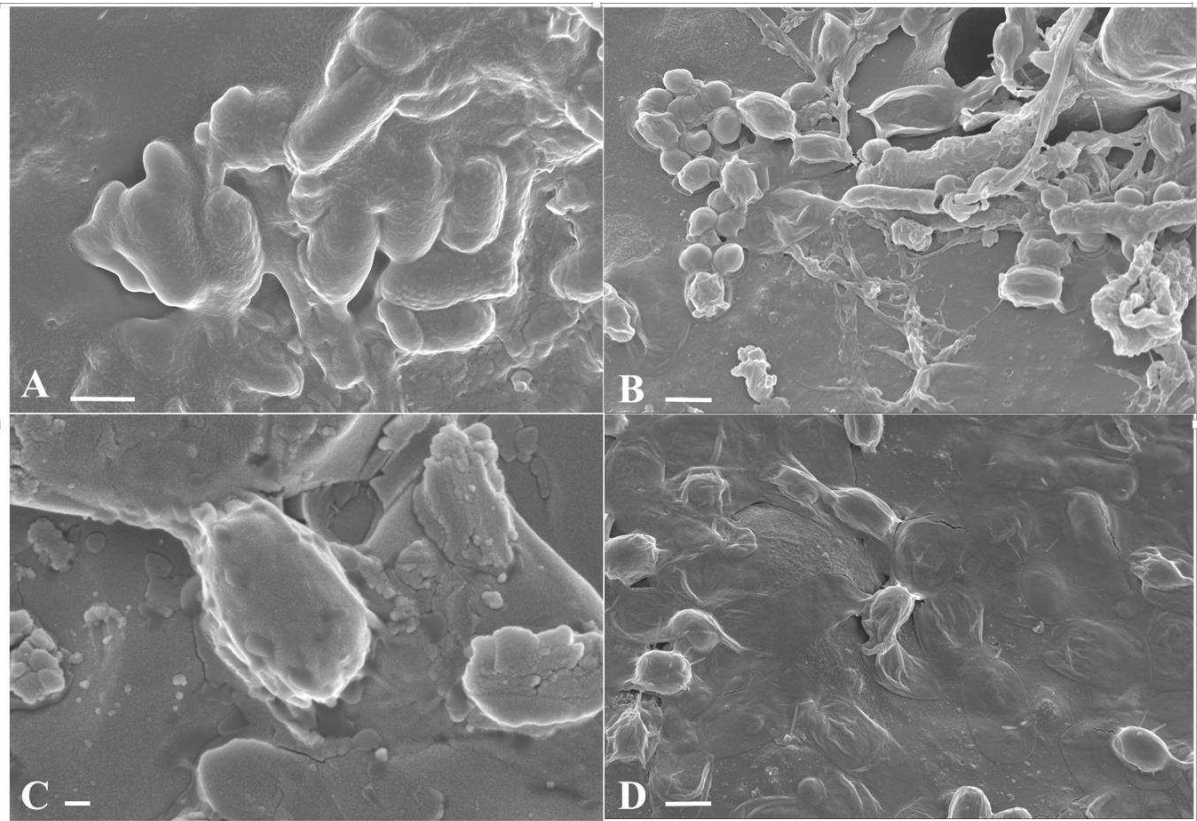
513

514 Figure 3: Recovery of unpurified and purified *C. difficile* spores from spiked clinical surfaces
 515 after treatment with 1000-ppm NaDCC for 10 min. Transfer testing was used to recover U and
 516 P spores of *C. difficile* strains (A) DS1748, (B) R20291 and (C) DS1813 from hospital surgical
 517 gowns after contact with: spores suspended in NaDCC applied to sterile gown, spiked gown
 518 exposed to NaDCC, spiked hospital stainless steel and hospital vinyl flooring exposed to
 519 NaDCC. The inoculum was used as the positive control (water only) and was also suspended
 520 in sodium thiosulfate to ensure no cross reactivity. Plots represent mean \pm SEM (n = 3).



521

522 Figure 4: Scanning electron micrographs of *C. difficile* spores present on spiked hospital
523 surgical gowns before and after treatment with NaDCC at 1,000-ppm for 10 min. Images depict
524 untreated (A) R20291 U spores on surgical gown fibres and (B) R20291 P single spore and
525 NaDCC treated (C) R20291 U spores on surgical gown fibres, (D) R20291 U single spore.
526 Arrows highlight spores adhered to gown fibres before (A) and after NaDCC treatment in (C),
527 and morphological changes in exosporium before (B) and after NaDCC treatment (D). Scale
528 bars in B and D are 200 nm, in A 2 μm , and in C 10 μm .



529

530 Figure 5: Scanning electron micrographs of *C. difficile* spores present on spiked hospital
531 stainless steel and floor vinyl before and after treatment with NaDCC at 1,000 ppm for 10 min.
532 Images are NaDCC-treated (A) DS1813 P spores on stainless steel; (B) DS1748 U Spores on
533 floor vinyl; (C) DS1748 U spores on stainless steel and (D) R20291 U spores on floor vinyl.
534 Arrows highlight areas in the exosporium layer. Scale bars in A, B and D are 1 μ m, and in C
535 200 nm.

536